



Autophosphorylation is crucial for CDK-activating kinase (Ee;CDKF;1) activity and complex formation in leafy spurge

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ABSTRACT

Ee;CDKF;1 protein is a leafy spurge (*Euphorbia esula*) CDK-activating kinase that is involved in a phosphorylation cascade linked to early stages of cell cycle progression. Yeast two-hybrid screening performed using Ee;CDKF;1 as a bait indicated that one of the interacting proteins was Ee;CDKF;1. Protein–protein interaction of Ee;CDKF;1 was further confirmed by yeast two-hybrid interaction and *in vitro* pull-down assays. Gel exclusion chromatography and/or native PAGE showed that GST-CDKF;1, MBP-CDKF;1, GST-CDKF;1 devoid of GST, and endogenous Ee;CDKF;1 were capable of forming homo protein complexes which are in dimer, trimer, and/or higher molecular-mass complex in its native state. In addition, Ee;CDKF;1 complexes were autophosphorylated and able to phosphorylate CDK. Moreover, mutant forms of Ee;CDKF;1 (106G/A, 166K/A), which lost autophosphorylation capability completely, were unable to form homo protein complexes in their native state. The result thus demonstrated that autophosphorylation of Ee;CDKF;1 is crucial for both kinase activity and complex formation.

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1. Introduction

Cell cycle progression is regulated by the sequential activation of cyclin-dependent kinases (CDKs) [1,2]. Binding of cyclins to CDKs is essential for CDK activity [3], whereas binding of CDK inhibitors (CKI) inhibits cell proliferation [4]. The activity of CDKs also depends on phosphorylation status, which is mediated by CDK-activating kinases (CAKs) [3].

CAKs are distantly related to CDKs and play important roles during cell cycle progression [5,6]. CAK phosphorylates a threonine (Thr) residue within the T-loop of CDKs [7,8]. Phosphorylation by CAK dramatically increases CDK's catalytic activity [7,9,10], while a loss of CAK activity is known to cause cell cycle arrest in yeast and *Drosophila* [11–15]. Another group of protein kinases, WEE1, execute inhibitory phosphorylation at Thr14 or Tyr15 of CDKs. Dephosphorylation of these residues by Cdc25 phosphatases is essential for CDK activity [16,17].

Eukaryotic organisms contain both heterotrimeric and monomeric CAK. Heteromeric CAK complexes are composed of three subunits: a catalytic subunit (p40^{MO15}/CDK7), a regulatory subunit (cyclin H) [18,19], and an assembly factor (MAT1) [20–23]. Heteromeric CAK complexes preferentially phosphorylate

CDK/cyclin complexes, which maximizes the kinase activity of CDK [18,24]. CDK7-cyclin H is also a component of the TFIIH complex that phosphorylates the C-terminal domain (CTD) of the largest subunit of RNA polymerase II [25–27]. In *Drosophila* and *Caenorhabditis elegans*, CDK7 activity is essential for survival [13,28]. Monomeric CAK (Cak1/Civ1p), first identified in budding yeast [11,12,15], phosphorylates CDK but lacks CTD kinase activity [12].

Plant CDKs are categorized into six types, i.e. A-type through F-type [29]. The A-type CDKs (CDKA;1) regulate the G1-to-S and G2-to-M transitions [30] and appear to play essential roles in embryogenesis and gametogenesis [31–33]. B-type CDKs regulate the G2-to-M progression [30,34], and divergent cellular functions are observed among different CDKBs. For example, CDKB1 appears to suppress endoreduplication [35] and CDKB2 seems to play a role in meristem organization [36]. C-type and E-type CDKs are not involved in cell division but play a role in transcription by phosphorylating the CTD of RNA polymerase II [37–39].

Apart from above-mentioned CDKs, most D- and F-types have CAK activity. The first CDK7/MO15 orthologue isolated from rice [40] was named R2 and Os;CDKD;1. This rice orthologue of CDK7 has both CDK and CTD kinase activities [41] and interacts with H-type cyclin (Os;cycH;1), which elevates both CDK and CTD activities. In Arabidopsis, At;CDKD;1, At;CDKD;2, and At;CDKD;3 (also named CAK3At, CAK4At, and CAK2At, respectively) were identified [42]. Whereas At;CDKD;1 lacks both CDK and CTD kinase activities and shows weak interaction with At;cycH;1 *in vitro* [43], At;CDKD;2 and At;CDKD;3 each individually bind with At;cycH;1 *in vitro* and cause increased CDK and CTD kinase activity [42,43]. However, the

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preference for substrates is not the same between At;CDKD;2 and At;CDKD;3; At;CDKD;2 has a higher CTD kinase activity and forms a stable complex with At;CycH;1 [44], whereas At;CDKD;3 has a higher CDK activity [42].

Arabidopsis CDKF (At;CDKF;1) was identified through rescue of budding yeast containing a CAK mutation [45]. In Arabidopsis cell suspensions, active At;CDKF;1 is a subunit of a 130-kD protein complex that phosphorylates At;CDKD;2 and At;CDKD;3 and activates the CTD kinase activity of At;CDKD;2 [43]. Based on the study of At;CDKF;1 knockout mutants, Takatsuka et al. [46] found that At;CDKF;1 plays a major role in regulating cell division, cell elongation, and endoreduplication. In leafy spurge, Ee;CDKF;1 (also named CAK1Ee) shares 63% identity and 75% similarity with At;CDKF;1. Both Ee;CDKF;1 and At;CDKF;1 consist of 480 amino acids, including a segment of about 110 amino acids between the kinase active site and the phosphoregulatory sites [47]. In addition, these two kinases have only CDK activity *in vitro* and do not interact with the H-type cyclin for kinase activity [43,45,47].

Previously, we found that two threonine residues (Thr291 and Thr296) of Ee;CDKF;1 were mutually responsible for intramolecular autophosphorylation and for phosphorylating CDK. In addition, Thr291 was constitutively autophosphorylated *in vivo* [47]. The other example of CDK autophosphorylation was found in a monomeric form of the human CDK2 [48]. This CDK2 was constitutively autophosphorylated at Thr160 and its kinase activity increased significantly after binding with cyclin A or E. Autophosphorylation is viewed as an example for efficient recruitment of substrate involved in protein phosphorylation [49]. While functional effects of autophosphorylation are not always detected in many protein kinases [50], we observed that the autophosphorylation of Ee;CDKF;1 is crucial for its substrate phosphorylation capacity. Since At;CDKF;1 is known to form a protein complex *in vivo*, in this study we used yeast two-hybrid technology to identify Ee;CDKF;1 interacting proteins and found that Ee;CDKF;1 binds to itself to form stable homo protein complexes in its native state. We also discuss the stability of monomeric Ee;CDKF;1, and the importance of autophosphorylation for protein complex formation.

2. Materials and methods

2.1. Yeast two-hybrid screening

Yeast strains and phagemid vectors in GAL4 Two-Hybrid Phagemid Vector Kits (Stratagene, La Jolla, CA) were used to screen a cDNA library in pAD-GAL4-2.1 phagemid vector, which was made from oligo (dT) linker-mRNA isolated from leafy spurge underground adventitious buds [51]. Manipulation of the yeast cells and library screening were carried out according to the manufacturer's instructions (Stratagene). In short, the complete coding region of Ee;CDKF;1 was PCR amplified using primer pair 1 for construct pBD-CDKF;1 (Table 1) and cloned into the *EcoRI* (5') and *Sall* (3') site (underlined sequences in oligonucleotides) of pBD-GAL4 Cam to make a pBD-CDKF;1 bait. The yeast YRG-2 reporter strain was first transformed with the pBD-CDKF;1 and subsequently with the leafy spurge cDNA library. Cells were plated onto 15-cm synthetic minimal (SD) plates (-Trp, -Leu, -His) to select for histidine prototrophy. The His⁺ colonies were re-streaked on selective medium and assayed for beta-galactosidase activity by filter assay. Crude yeast DNA preparations were used to transform *E. coli* strain XL1-Blue and selected for target or bait plasmid by plating on LB-ampicillin or LB-chloramphenicol agar plates, respectively. Clones were sequenced (DNA Facility in Iowa State University) and analyzed using Lasergene software (DNASTAR Inc., Madison, WI).

2.2. Ee;CDKF;1 protein–protein interaction studies

For protein–protein interaction, a full-length Ee;CDKF;1 was amplified using primer pair 2 for construct pAD-CDKF;1 (Table 1) containing *EcoRI* and *XhoI* restriction sites. Pfu DNA polymerase (Stratagene, La Jolla, CA) was used in PCR reactions. PCR products were digested with *EcoRI* and *XhoI* and ligated into the *EcoRI* (5') and *XhoI* (3') sites of pAD vector and the construct was verified by sequencing. Yeast strain YRG-2 was transformed with pAD-CDKF;1, pBD-CDKF;1, pAD, or pBD to examine if Ee;CDKF;1 has auto-activation capacity. This yeast was also co-transformed with pAD-CDKF;1 + pBD, pBD-CDKF;1 + pAD, or pAD-CDKF;1 + pBD-CDKF;1 to examine if Ee;CDKF;1 interacts with Ee;CDKF;1 molecules. At least 10 individual clones were obtained from each transformant containing the desired plasmid(s). These transformants were grown on SD plates lacking Leucine (-Leu), Tryptophan (-Trp), Tryptophan and Leucine (-Trp, -Leu), or Tryptophan, Leucine, and Histidine (-Trp, -Leu, -His).

2.3. GST pull-down experiments

Several constructs were made for *in vitro* pull-down assays. A histidine-tagged fusion Ee;CDKF;1 was made by PCR amplifying the complete coding region of Ee;CDKF;1 using primer pair 3 for construct pHis₆-CDKF;1 (Table 1). The fragment was then cloned into the *BamHI-Sall* site of pQE30 vector (Qiagen, Valencia, CA) to generate a pHis₆-CDKF;1. A full-length GST-tagged fusion Ee;CDKF;1 was also made by PCR-based cloning using primer pair 4 for construct pGST-CDKF;1 (Table 1). This fragment was cloned into the *EcoRI* (5') and *XhoI* (3') site of pGEX-4T-1 vector (Amersham Biosciences, Piscataway, NJ). pHis₆-CDKF;1 was co-transformed with a pGEX-4T-1 (control plasmid) or pGST-CDKF;1 into *E. coli* BL21 star cells (Invitrogen, Carlsbad, CA). Plasmid DNAs were isolated from these transformants to confirm that both His₆ and GST plasmids were in the same cell using PCR. The cells were grown in Luria-Bertani medium until the OD₆₀₀ reading = 0.5 and induced with isopropyl-1-thio-β-D-galactopyranoside (IPTG) at the final concentration of 0.1 mM for 3 h at 37 °C. Cells were centrifuged at 5000 × g for 5 min and the pellet was resuspended in 1 × Phosphate Buffered Saline (PBS, 137 mM NaCl, 10 mM Na₂HPO₄, 2.7 mM KCl, 18 mM KH₂PO₄, pH 7.4). Cells were lysed on ice with a Branson Sonifier 450 (Branson, Danbury, CT) with the output control setting at 1, a duty cycle setting of constant, and 6 sonication pulses of 20 s per pulse. The lysate was centrifuged for 10 min at 10,000 × g. The supernatant was incubated with Glutathione-Sepharose 4B beads for 30 min at 4 °C and washed 3 times with 500 μL ice-cold 1 × PBS buffer with 10 min intervals between each wash. The protein was eluted with Glutathione Elution Buffer (GEB) and examined on a 10% SDS (w/v) polyacrylamide gel. Western blotting was performed according to the standard procedures using penta-His antibody (Qiagen) and GST antibody (Pharmacia) at a 1:200 dilution. Secondary antibodies were anti-mouse IgG-conjugated with horseradish peroxidase (Bio-Rad) and anti-goat IgG-conjugated with alkaline phosphatase at 1:10,000 dilutions against His and GST proteins, respectively. The blots were developed using a SIGMA FAST™ BCIP/NBT kit according to manufacturer's instructions (Sigma–Aldrich, St. Louis, MO).

2.4. Gel exclusion chromatography

GST-CDKF;1 fusion protein was affinity-purified using Glutathione Sepharose 4B (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions. To obtain a CDKF;1 protein devoid of GST, thrombin was used to cleave purified GST-CDKF;1 fusion protein according to the manufacturer's instructions. Proteins were separated by gel exclusion chromatography on a HiLoad 16/60 Superdex 200 fast protein liquid chromatography col-

Table 1

Primers used to amplify inserts for cloning purpose.

Construct name	Forward primer 5'–3'	Reverse primer 5'–3'
1. pBD-CDKF;1	TAATAGA <u>GAATTC</u> ATGAGCAGTTACCGCCATC	ACTGTCGACTCATCAGGAAACTGTATGGAGAAA
2. pAD-CDKF;1	TAATAGA <u>GAATTC</u> ATGAGCAGTTACCGCCATCT	ACTCTCGAGTCATCAGGAAACTGTATGGAGAAA
3. pHis ₆ -CDKF;1	TAATAGGATCCATGAGCAGTTACCGCCATC	ACTGTCGACTCATCAGGAAACTGTATGGAGAAA
4. pGST-CDKF;1	TAATAGA <u>GAATTC</u> ATGAGCAGTTACCGCCATCT	ACTCTCGAGTCATCAGGAAACTGTATGGAGAAACC

Underlined sequences, GAATTC, GGATCC, GTCGAC, and CTCGAG, represent *EcoRI*, *BamHI*, *Sall*, and *XhoI* sites, respectively.

umn (Pharmacia Biotech, Uppsala, Sweden). The column was first equilibrated with several column volumes of gel filtration buffer (100 mM KCl, 50 mM Tris-aminomethane, pH 8.0) at a flow rate of 0.5 mL min⁻¹. One mg, in a volume of 0.5 mL, affinity-purified and thrombin-digested GST-CDKF;1 was then loaded onto the equilibrated column and the chromatography was performed at the same flow rate, collecting 1 mL fractions. Fractions were analyzed by SDS PAGE gel, immunoblot analysis, and kinase assays according to the methods described previously [47].

2.5. Immunoaffinity purification of endogenous Ee;CDKF;1

Ee;CDKF;1 antibody (PA) was immobilized to Protein G using the Protein G Immunoprecipitation Kit (Thermo Scientific, Prod #45210, Rockford, IL) according to manufacturer's instructions. Seedling extract was run through a 1 cm Bio-Rad column packed with the immobilized Ee;CDKF;1 antibody to form the immune complex. Endogenous Ee;CDKF;1 was eluted with low pH (0.1 M glycine-HCl, pH 2.8, Thermo Scientific IgG Elution Buffer, Prod #21004), ionic strength (pH 6.6, Thermo Scientific Gentle Ag/Ab Elution Buffer, Prod #21027), or high pH (0.1 M glycine-NaOH, pH 10) buffer. Fractions (100 µL) of low and high pH eluates were neutralized immediately with 5 µL of 1 M Tris at pH 8.8 and pH 6, respectively. Two mL Zeba Spin Desalting Columns (Thermo Scientific, Prod #89890) were used to remove salts and aggregated (denatured) Ee;CDKF;1 from pooled eluate. The desalted samples were then concentrated using a SpeedVac concentrator.

2.6. Protein extraction, immunoblot analysis, and native and SDS PAGE

MPB-Ee;CDKF;1 and GST-CDKF;1 were affinity purified according to the method of Chao et al. [47]. Ee;CDKF;1 polyclonal antibody (PA) was described previously [47]. Native PAGE analysis was performed using the NativePAGE™ Novex® Bis-Tris Gel system (Invitrogen, Carlsbad, CA), which uses Coomassie® G-250 as a charge-shift molecule. SDS PAGE analysis was carried out according to the method of Laemmli [52] using a 10% polyacrylamide resolving gel and a 4% stacking gel. The immunoblot procedures were described by Wang et al. [53].

3. Results

3.1. Yeast two hybrid assays

A yeast two-hybrid system was used to determine possible protein–protein interaction of Ee;CDKF;1. After screening 1.4×10^8 clones using pBD-CDKF;1 as a “bait”, many positive cDNA clones including Ee;CDKF;1 (CDK-activating kinase), cyclin D3-2, histone H3, and others (see [Supplementary Table 1](#)) were identified. The finding that Ee;CDKF;1 interacted with Ee;CDKF;1 molecules was unanticipated, but the result could explain how molecular weight of the native Arabidopsis CDKF;1 (At;CDKF;1), a functional homologue of Ee;CDKF;1, is around 130-kD [43].

Previous work showed that Ee;CDKF;1 consists of 480 amino acids with an estimated molecular weight of 54-kD. In addition, similar to the findings of Umeda et al. [45], Ee;CDKF;1 does not require other proteins for activity [47]. To confirm the assumption that Ee;CDKF;1 forms homo protein complexes, yeast two-hybrid assays were performed.

[Fig. 1](#) shows that yeast strain YRG-2 harboring pAD-CDKF;1 or pAD (#3, #6) grew well on synthetic medium (SD) lacking Leucine (-Leu), but did not grow on the SD medium lacking Tryptophan (-Trp), lacking Tryptophan and Leucine (-Trp, -Leu) or lacking Tryptophan, Leucine and Histidine (-Trp, -Leu, -His). In contrast, yeast strain YRG-2 harboring pBD or pBD-CDKF;1 (#2, #5) grew well on synthetic medium (SD) lacking Tryptophan (-Trp), but did not grow on the SD medium lacking Leucine (-Leu), lacking Tryptophan and Leucine (-Trp, -Leu) or lacking Tryptophan, Leucine and Histidine (-Trp, -Leu, -His). The above results indicated that Ee;CDKF;1 were correctly inserted into these two yeast vectors and Ee-CDKF;1 has no auto-activation capacity. YRG-2 harboring the combination of pBD-CDKF;1 and pAD (#1) grew well on SD (-Trp, -Leu) plates but did not grow on SD (-Trp, -Leu, -His) plates. Likewise, YRG-2 harboring the combination of pAD-CDKF;1 and pBD grew well on SD (-Trp, -Leu) plates but did not grow on SD (-Trp, -Leu, -His) plates ([Supplementary Fig. 1](#)). These results indicated that YRG-2 contained both plasmid vectors, but target and bait proteins did not interact. However, YRG-2 harboring the combination of pAD-CDKF;1 and pBD-CDKF;1 (#4) grew well on all four types of SD plates. The fact the YRG-2 could grow on SD (-Trp, -Leu, -His) plates indicated that the full-length Ee;CDKF;1 interacted with another full-length Ee;CDKF;1.

3.2. In vitro pull-down assays

Interaction of Ee;CDKF;1 molecules was further confirmed by Glutathione S-transferase (GST) pull-down assays. In this experiment, bacteria cells were co-transformed with a pHis₆-CDKF;1 plasmid and a pGEX-4T-1 (control plasmid) or pGST-CDKF;1. After IPTG induction, GST fusion proteins were affinity-purified using a Glutathione Sepharose 4B column. If Ee;CDKF;1 interacts with another Ee;CDKF;1, both the bait (GST-CDKF;1) and prey (His₆-CDKF;1_{1–1443}) proteins can be eluted and identified by anti-GST and anti-His₆ antibodies, respectively.

Duplicate blots of pull-down products were incubated with anti-His₆ and anti-GST antibodies separately ([Fig. 2](#)). Anti-His₆ antibody recognized a single 54-kD His₆-CDKF;1 protein that was affinity purified with Nickel Chelated Column from bacteria harboring only pHis₆-CDKF;1 plasmid (lane 1, blot A); likewise, anti-GST antibody recognized a single 80-kD GST-CDKF;1 protein that was affinity purified with Glutathione-Sepharose 4B beads from bacteria harboring only pGST-CDKF;1 plasmid (lane 4, blot B). When bacteria cells were co-transformed with a pHis₆-CDKF;1 plasmid and a pGEX-4T-1 plasmid, and proteins were purified with a Glutathione Sepharose 4B column, only a 26-kD GST protein was identified using anti-GST antibody (lanes 2, blot B); no band was identified using anti-His₆ antibody (lane 2, blot A), indicating that GST did not interact with His₆ or Ee;CDKF;1. In contrast,

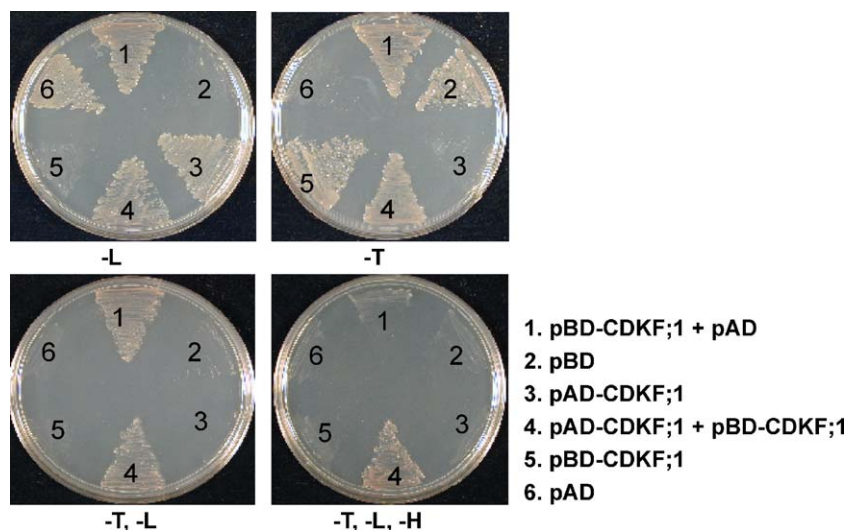


Fig. 1. Yeast two-hybrid analysis. The yeast strain YRG-2 (Stratagene, La Jolla, CA) was transformed with pBD (#2, GAL4 binding domain), pAD (#6, GAL4 activation domain), pAD-CDKF;1 (#3, AD + Ee;CDKF;1), or pBD-CDKF;1 (#5, BD + Ee;CDKF;1) according to Stratagene two-hybrid protocol. This yeast strain was also co-transformed with pBD-CDKF;1 and pAD (#1) or with pAD-CDKF;1 and pBD-CDKF;1 (#4). Transformed yeast cultures with an OD₆₀₀ of 0.025 were used for growth plating assays on selection medium plates, lacking Leucine (-L), Tryptophan (-T), Leucine and Tryptophan (-L, -T), or Leucine, Tryptophan, and Histidine (-L, -T, -H).

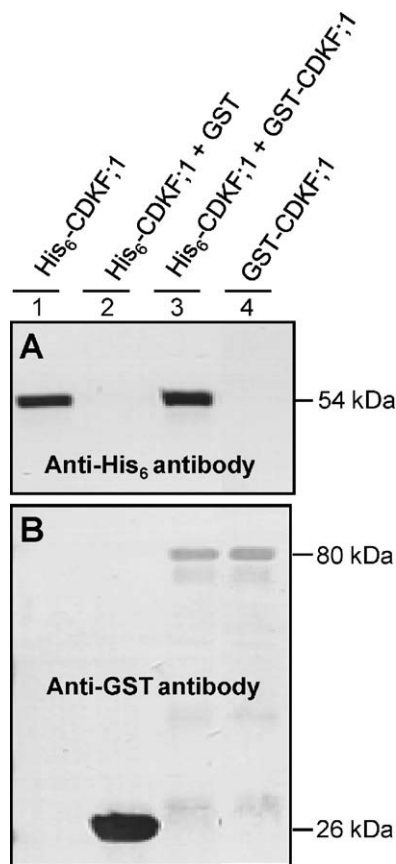


Fig. 2. *In vitro* pull-down analysis. *E. coli* strain BL21 star (Invitrogen, Carlsbad, CA) was transformed with pHis₆-CDKF;1 or pGST-CDKF;1. BL21 star was also co-transformed with a pHis₆-CDKF;1 plasmid and a pGEX-4T-1 or pGST-CDKF;1. Lanes 2 and 3 are proteins that were affinity-purified with Glutathione-Sepharose 4B beads, separated on a 10% SDS (w/v) PAGE gel, blotted onto membranes, and incubated with anti-His₆-antibody (A) or anti-GST-antibody (B). Lanes 1 and 4 are His₆-CDKF;1 and GST-CDKF;1 protein controls that were affinity purified with Nickel Chelated Column and Glutathione-Sepharose 4B beads, respectively.

when bacteria cells were co-transformed with a pHis₆-CDKF;1 plasmid and a pGST-CDKF;1 plasmid and subjected to protein purification with a Glutathione Sepharose 4B column, anti-His₆ antibody recognized a 54-kD His₆-CDKF;1 protein (lane 3, blot A) and anti-GST antibody recognized a GST-CDKF;1 protein of 80-kD (lane 3, blot B). *In vitro* pull-down assays combined with yeast two-hybrid results clearly showed that the full-length Ee;CDKF;1 interacted with Ee;CDKF;1 molecules.

3.3. Ee;CDKF;1 forms homo protein complexes

The molecular weight of the Ee;CDKF;1 complex was determined by Superdex 200 gel exclusion chromatography (Fig. 3). GST-CDKF;1 protein was affinity-purified, digested with thrombin, and fractionated using a gel exclusion column. Two separate chromatographs are shown in Fig. 3A (top right corner); peaks a through f are protein standards and peaks 1 and 2 are thrombin-digested GST-CDKF;1. A linear relationship is obtained after plotting the logarithm of protein standard molecular weight versus V_e/V_0 . Based on this graph, the predicted molecular weight of peak 1 is ~133-kD and peak 2 is ~54-kD. Fractions from peak 1 (fractions 1–7) and peak 2 (fractions 8–14) were separated by SDS PAGE and stained with Coomassie Brilliant Blue (Fig. 3B). In this figure, intact GST-CDKF;1 has one major 80-kD band. Thrombin digested GST-CDKF;1 (TD) has 3 major bands (54-, 40-, and 26-kD). The rest of the protein lanes are fractions collected after running thrombin-digested GST-CDKF;1 through gel exclusion column. A 54-kD doublet and 40-kD bands were identified from peak 1, fractions 1–5. Since Ee;CDKF;1 can interact with itself, it is possible that the 54-kD doublet observed in Fig. 3B may correspond to both phosphorylated and non-phosphorylated forms of Ee;CDKF;1 or, alternatively, partial thrombin digestion prior to ATP-binding site (Gly36) could also explain the doublet. The 40-kD band is a partial Ee;CDKF;1. We speculate that it was digested by thrombin through a non-optimum cleavage site. A 54-kD Ee;CDKF;1 and a 26-kD GST were identified between peak 1 and 2 (fractions 6–9). The 26-kD band is the denatured form of GST which forms homo-dimer *in vivo* [54]. Only 26-kD GST was identified from peak 2, fractions 10–14.

Peaks 1 and 2 were examined further based on immunoblot analyses and kinase assays (Fig. 4). Immunoblot analyses using

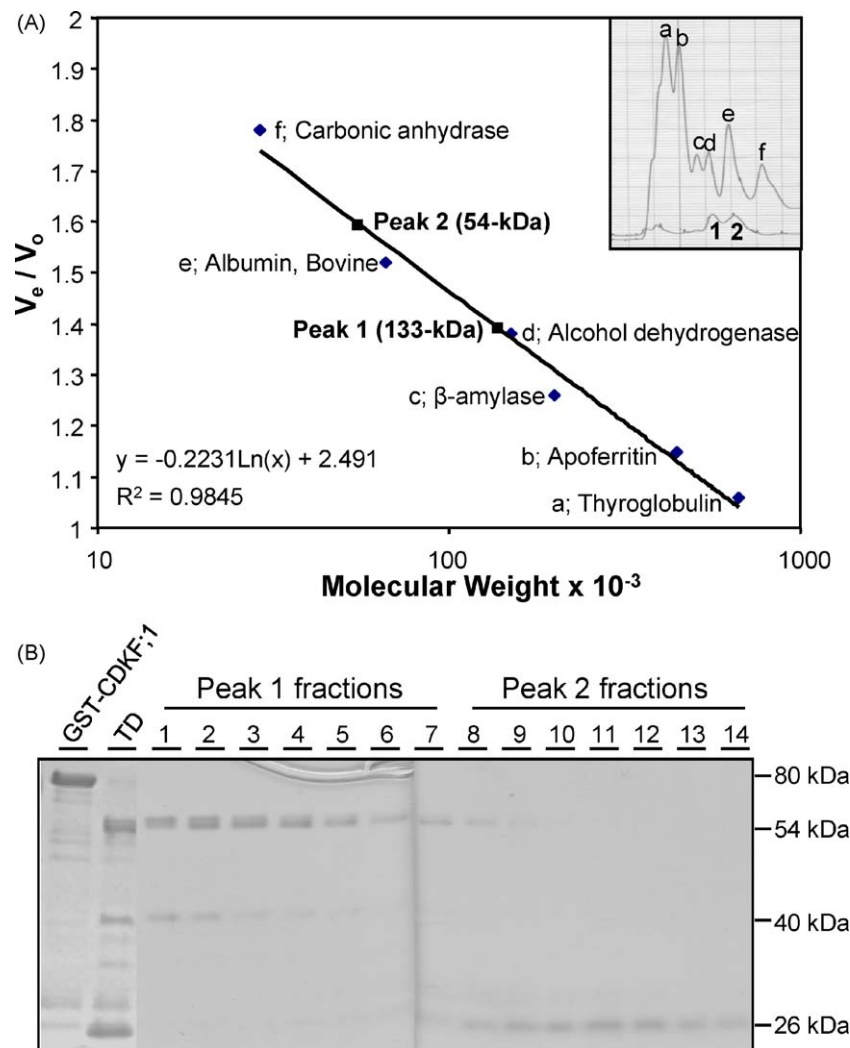


Fig. 3. Analysis of thrombin-digested GST-CDKF;1 using gel exclusion chromatography. (A) Gel exclusion chromatography analysis. GST-CDKF;1 was column-purified, digested with thrombin, and fractionated using gel exclusion chromatography. Peaks a–f are protein standards and peaks 1 and 2 are thrombin-digested GST-CDKF;1. (B) SDS PAGE analysis. Protein samples of peaks 1 and 2 were fractionated on SDS PAGE. GST-CDKF;1 was a fusion protein prior to thrombin digestion. TD was thrombin digested GST-CDKF;1 prior to running through gel exclusion chromatography. Numbers 1–14 were fractions collected during gel exclusion chromatography analysis.

Ee;CDKF;1 polyclonal antibody detected the 54-kD Ee;CDKF;1 doublet and the 40-kD band, but not the 26-kD GST (Fig. 4A). Kinase assays showed that the 54-kD Ee;CDKF;1 doublet was autophosphorylated, and it also phosphorylated its substrate protein, the 45-kD CDK (Fig. 4B). The 40-kD band is faintly phosphorylated, indicating that deletion of the carboxyl terminus may partially inhibit Ee;CDKF;1 autophosphorylation. Since the estimated molecular weight of peak 1 is 133-kD (ranging from 100- to 165-kD), and it contains 54-kD and 40-kD Ee;CDKF;1 molecules, the combinations of Ee;CDKF;1 complexes could be 160-kD tetramer (4 40-kD molecules), 162-kD trimer (3 54-kD molecules), 148-kD trimer (2 54-kD molecules + 1 40-kD molecule), 134-kD trimer (1 54-kD molecule + 2 40-kD molecules), 120-kD trimer (3 40-kD molecules), and 108-kD dimer (2 54-kD molecules) at various percentages. No monomeric form of Ee;CDKF;1 was observed from any fractions of peaks 1 and 2.

3.4. Autophosphorylation required for protein–protein interaction of Ee;CDKF;1

Previously, we transformed wild and mutant forms of Ee;CDKF;1 into a budding yeast CAK mutant, *Saccharomyces cerevisiae* GF2351.

GF2351 is a conditional CAK mutant that grows normally at 25 °C but does not grow at 37 °C. We identified that a mutation at 106G/A (at Gly36 of the ATP-binding site) or 166K/A (at Lys56 of the ATP-aligning site) of Ee;CDKF;1 severely inhibited yeast (GF2351) growth and totally abolished the ability of Ee;CDKF;1 to autophosphorylate and phosphorylate CDK [47]. In addition, mutation at either 871T/A (at Thr291 of the phosphoregulatory site) or 886T/A (at Thr296 of the phosphoregulatory site) drastically reduced both auto and substrate phosphorylation since these two threonine residues are mutually responsible for intra-molecular autophosphorylation and for phosphorylating its substrate protein, cyclin-dependent kinase (CDK) [47]. To determine if autophosphorylation is required for protein–protein interaction, wild type GST-CDKF;1 and 4 GST-CDKF;1 mutant proteins (106G/A, 166K/A, 871T/A, and 886T/A) were fractionated on a native PAGE (Fig. 5A–1). The results showed that wild type GST-CDKF;1 (lane 1) had two bands; one was around 150-kD and the other was around 260-kD. The two threonine to alanine substitution mutants (886T/A and 871T/A) also had two bands with similar mobility as the wild type GST-CDKF;1 (lanes 4 and 5, see arrows); however, the levels of these two protein complexes were significantly lower than their wild type counterpart. In contrast,

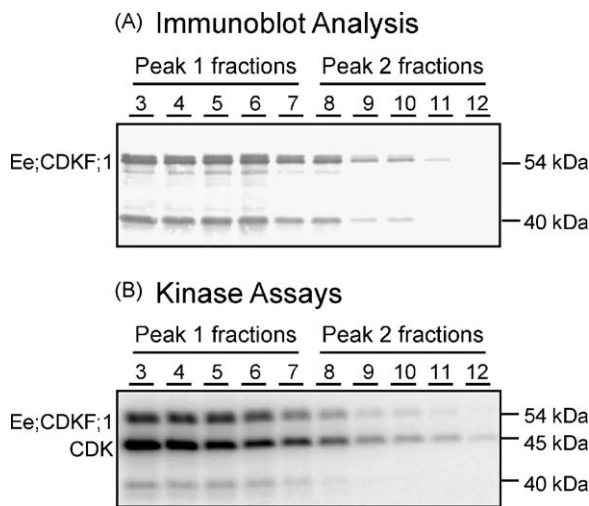


Fig. 4. Immunoblot and kinase analysis. GST-CDKF;1 was column-purified, digested with thrombin, and fractionated using gel exclusion chromatography. Proteins (fraction numbers 3 through 12) of peaks 1 and 2 were fractionated by SDS PAGE, blotted onto membranes, and incubated with PA. (A) These proteins were also used for kinase assays. (B) For immunoblot analysis, 62 ng per lane was loaded on SDS PAGE. For each kinase assay, 26 ng of protein fraction was used.

no protein band was observed for GST-CDKF;1 mutated at the ATP-binding site (lane 2) or ATP-aligning site (lane 3) although these two proteins were readily shown using SDS PAGE (Fig. 5A–2), indicating that autophosphorylation is crucial for protein complex formation.

Analogous results were obtained using MBP-CDKF;1 mutant proteins (Fig. 5B). Wild type MBP-CDKF;1 formed two bands on a native PAGE, but these two protein bands were decreased considerably for MBP-CDKF;1 mutated at the ATP-binding site (lane 2) and virtually absent at the ATP-aligning site (lane 3). The two threonine to alanine substitution mutants (886T/A and 871T/A) also had two bands with similar mobility as the wild type MBP-CDKF;1 (lanes 4 and 5, see arrows); however, the levels of the larger protein complex were significantly reduced. Native PAGE analysis indicated that a mutation at the ATP-binding site or ATP-aligning site of Ee;CDKF;1 block protein complex formation. Since the monomeric form of mutant Ee;CDKF;1 was practically absent, we assume that

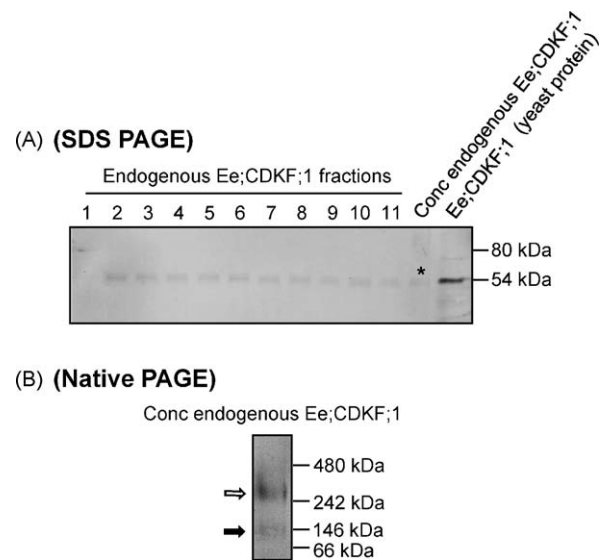


Fig. 6. Native PAGE analysis of immunoaffinity purified endogenous Ee;CDKF;1. The seedling extract was run through a Bio-Rad column packed with the immobilized Ee;CDKF;1 antibody, and the proteins were eluted with high pH buffer. Fractions #1 through 11 are eluates representing endogenous Ee;CDKF;1. Conc endogenous Ee;CDKF;1 was pooled, desalted and concentrated endogenous Ee;CDKF;1 fractions #1–11. Ee;CDKF;1 (yeast protein) is the protein extracted from yeast harboring the pXY-Ee;CDKF;1 plasmid [47]. Proteins were fractionated by SDS PAGE (A: upper blot) or native PAGE (B: lower blot), blotted onto membranes, and incubated with Ee;CDKF;1 antibody. Twelve μ L was loaded for native PAGE and 30 μ L was loaded for SDS PAGE. The protein level of pooled, desalted, and SpeedVac dried endogenous Ee;CDKF;1 is indicated by asterisk on the SDS PAGE gel. Homo protein complexes of endogenous Ee;CDKF;1 are indicated as closed and open arrow on the native gel.

it formed aggregates and was not able to migrate in a native gel.

3.5. Endogenous Ee;CDKF;1 forms protein complexes as expected

Endogenous Ee;CDKF;1 can be eluted by three types of buffers as described in Section 2. The protein fractions eluted by high pH buffer worked best and can be observed in SDS PAGE directly after incubating the blots with PA (Fig. 6A, fractions 1–11). When fractions 1–11 were pooled, desalted, and SpeedVac dried to less than

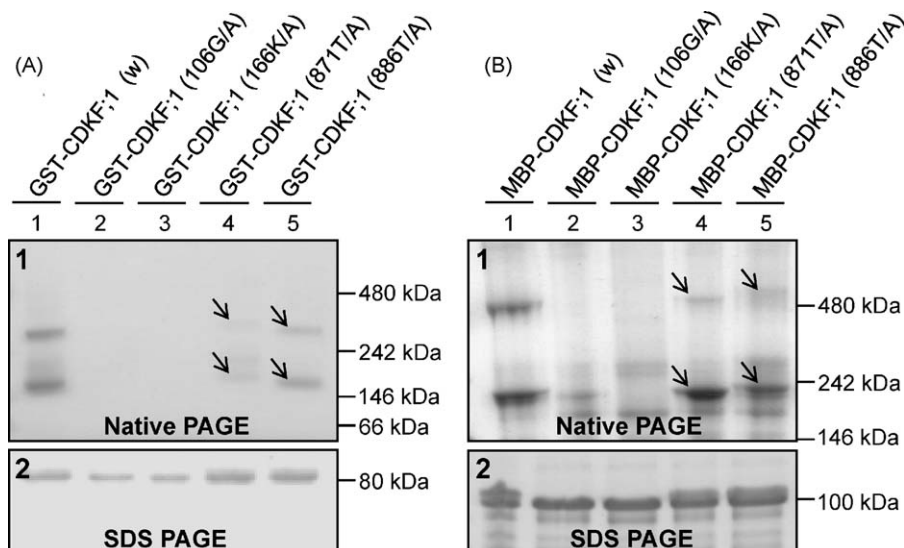


Fig. 5. Native PAGE analysis of Ee;CDKF;1 mutants. Wild-type (W) and mutant (106G/A, 166K/A, 871T/A, and 886T/A) GST-CDKF;1 (A) and MBP-CDKF;1 (B) were column-purified and fractionated by native PAGE (1: upper gel). The quality and quantity of these proteins were examined by SDS PAGE (2: lower gel). Three μ g per lane was loaded for native and SDS PAGE.

1/10 volume, an increase in protein levels was not observed by this concentration protocol as the intensity of this protein band is similar to or less than non-concentrated fractions 1–11 in SDS PAGE (see Fig. 6A, asterisk), indicating that the desalting procedure using Zeba Spin Desalting Column not only reduced salt concentration but also removed denatured, aggregated forms of endogenous Ee;CDKF;1.

For native PAGE, no protein bands were observed for fractions 1–11 (data not shown). In contrast, two bands can be seen in native PAGE for the pooled, desalted, and SpeedVac dried sample (see Fig. 6B, closed and open arrows) at MWs of ~140-kD and ~250-kD. The 140-kD protein complex observed in Fig. 6B is similar in size to the 133-kD protein complex (peak 1) observed by gel filtration in Fig. 3A. However, the 250-kD protein complex observed in Fig. 6B does not correspond to any protein complexes separated by gel filtration. As previously discussed at the end of Section 3.3, the 250-kD protein complex could represent multiple combinations of tetrameric Ee;CDKF;1. Alternatively, since Ee;CDKF;1 interacts with other proteins (see Supplementary Table 1) and a 250-kD protein complex was not observed during gel filtration, we assume that Ee;CDKF;1 likely interacts with other unknown proteins *in vivo* to account for the larger protein complex observed in Fig. 6B.

4. Discussion

Our data confirm that Ee;CDKF;1 indeed interacts with other Ee;CDKF;1 based on both yeast two-hybrid and *in vitro* pull-down analysis. In addition, Ee;CDKF;1 forms homo protein complexes in its native state based on gel exclusion chromatography and native gel electrophoresis. However, an intriguing question is why Ee;CDKF;1 interacted with so many other proteins during yeast two-hybrid screening if it forms a stable homo protein complex. One explanation could be that the monomeric form of Ee;CDKF;1 is highly interactive, which would form protein complexes readily with other cellular proteins if it did not form a homo protein complexes in time.

We also found that pull-down assays did not work by simply incubating the prey (His₆-CDKF;1) and bait (GST-CDKF;1) proteins together (data not shown), indicating that as soon as Ee;CDKF;1 was synthesized, it likely forms a stable complex with other Ee;CDKF;1 molecules, and once a complex is formed, Ee;CDKF;1 molecule is no longer interchangeable among neighboring analogous complexes. In fact, Ee;CDKF;1 may be very unstable in its monomeric form because no monomeric Ee;CDKF;1 was identified from our gel exclusion chromatography and native gel electrophoresis. From this observation, we can assume that protein–protein interaction for Ee;CDKF;1 is so fast *in vivo* that the chances of forming protein complexes with other protein molecules are minimal. Alternatively, some chaperone-like proteins such as Cdc37 and Hsp90 [55] may be required to maintain correct Ee;CDKF;1 folding before interacting with other molecules. In budding yeast *S. cerevisiae*, Cdc28 (Cell division control protein 28) and Cak1 require Cdc37 to maintain stability, and the presence of Cdc37 increases Cak1 production and activity [56].

Our results also indicate that denatured forms of Ee;CDKF;1 formed aggregates instantly and did not migrate in the native PAGE gel. In contrast, the endogenous Ee;CDKF;1 complexes are clearly shown on native gels (Fig. 6B, conc endogenous Ee;CDKF;1) using the pooled, desalted, and concentrated eluate. However, the intensity of this protein band was not increased in SDS PAGE after increasing the concentration of eluate to 10-fold by drying the eluate to 1/10 of the pooled volume (Fig. 6A, conc endogenous Ee;CDKF;1). This result further confirmed that the elution process denatured most of the endogenous Ee;CDKF;1 (over 90%), which was likely aggregated and trapped in the column during the desalting process. We also found that it is important to use

freshly purified endogenous Ee;CDKF;1 fractions for desalting and SpeedVac concentration since protein smearing problem worsened if these fractions were stored at –80 °C, indicating that denaturing process for native Ee;CDKF;1 continued in neutralized elution buffer used in this study.

We have shown that Ee;CDKF;1 autophosphorylation is crucial for its kinase activity and can be totally abolished by mutation at the ATP-binding site (106G/A) or ATP-aligning site (166K/A) [47]. The current study further indicated that autophosphorylation is crucial for forming homo protein complexes, and which reaction should occur prior to forming a protein complex as no complex was observed if either ATP-binding or ATP-aligning capability was removed (Fig. 5). Although Ee;CDKF;1 does not require both threonine residues (Thr291 and Thr296) to form a stable complex, the amount of complex production is compromised; a significant reduction in protein levels is observed (Fig. 5). Thus, the phosphorylation of any one of these two threonine residues may facilitate Ee;CDKF;1 to form a tertiary structure for complex formation; however, to achieve a full potential, both threonine residues need to be phosphorylated.

In summary, we found that Ee;CDKF;1 can interact with other Ee;CDKF;1 homo protein to form dimer, trimer, and/or higher molecular-mass complexes. More importantly, it requires autophosphorylation to form protein complexes. Previously, we identified that Thr291 is constitutively phosphorylated *in vivo*, which makes Ee;CDKF;1 steadily competent to activate CDKs. The Arabidopsis At;CDKF;1 is a functional homolog of Ee;CDKF;1. At;CDKF;1 also forms an active 130-kD complex, which is likely to be the combination of homo dimer and trimer. The fact that both Ee;CDKF;1 and At;CDKF;1 form a 130-kD homo protein complex and are activated by autophosphorylation allows us to propose that both CDKF;1s are at or near the top of their kinase pathways. When CDKF;1s are synthesized *in vivo*, these molecules autophosphorylate and form homo protein complexes immediately. CDKF;1

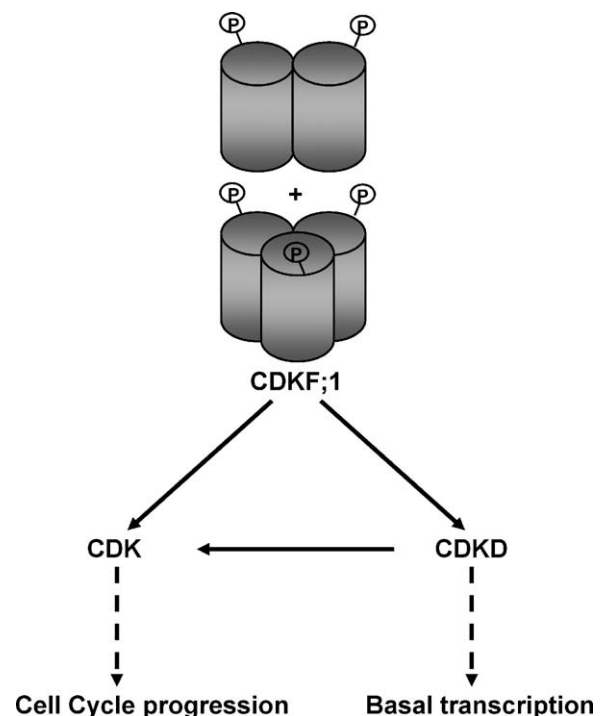


Fig. 7. CDKF;1 autophosphorylation and activity. CDKF;1 may be at/near the top of its kinase pathways. After CDKF;1s are synthesized *in vivo*, these molecules autophosphorylate and form homo protein complex immediately. CDKF;1 complex is then able to phosphorylate and activate CDKs during cell cycle progression and to activate other CDKD proteins as Shimotohno et al. [43] have shown for At;CDKF;1.

complex is then able to phosphorylate and activate CDKs during cell cycle progression and to activate other CAK proteins as Shimotohno et al. [43] have shown for AtCDK1 (Fig. 7). Although CDK1 functions at/near the top of a phosphorylation cascade, our data has not provided direct evidence indicating that CDK1 is the master regulator of this pathway. It could be that the process of forming homo-dimers/trimers regulates downstream pathways. It is also possible that the rate determining steps of cell cycle are controlled by downstream proteins such as CDKs, cyclins, etc, and steady competence of CDK1 complex could allow it to partake in cellular activity efficiently. Such an effect could contribute to accelerate the recovery of meristematic activity. Interestingly, our data also clearly showed that EeCDK1 has the capacity to form complexes with other proteins (see Supplementary Table 1); thus, further studies will be required to determine how protein–protein interaction of EeCDK1 affects its ability to form complexes and phosphorylate other downstream cell cycle regulating proteins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2010.08.017.

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